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## Request for grant of a patent

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| 2. | Patent applicati *(The Patent office v* | 9719142.3 09 SEP 1997 |
| 3. | Full name, address and postcode of the or of each applicant *(underline all surnames)* | GLAXO GROUP LIMITED<br>GLAXO WELLCOME HOUSE<br>BERKELEY AVENUE<br>GREENFORD<br>MIDDLESEX<br>UB6 ONN<br>GB |
| | Patents ADP number *(if you know it)* | |
| | If the applicant is a corporate body, give the country/state of its corporation | GB 4735870003 |
| 4 | Title of the invention | ANALYTICAL METHOD AND APPARATUS THEREFOR |
| 5 | Name of your agent *(if you know one)* | RACHEL M. THORNLEY (SEE CONTINUATION SHEET) |
| | "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* | GLAXO WELLCOME PLC<br>GLAXO WELLCOME HOUSE, BERKELEY AVENUE<br>GREENFORD, MIDDLESEX<br>UB6 ONN, GB |
| | Patents ADP number *(if you know it)* | 07625400 1 |

| 6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number | Country | Priority application number *(if you know it)* | Date of Filing (day / month / year) |
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| 7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application | Number of earlier application | Date of filing (day / month / year) |
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| 8. | Is a statement of inventorship and of right to grant a patent required in support of this request? *(Answer yes if:*<br>*a) any applicant named in part 3 is not an inventor, or*<br>*b) there is an inventor who is not named as an applicant, or*<br>*c) any named applicant is a corporate body.* | YES |

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| Priority Documents | |
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| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
| Request for preliminary examination and search *(Patent Form 9/77)* | |
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11. **I/We request the grant of a patent on the basis of this application**

Signature **RACHEL M. THORNLEY** **9 September, 1997**

***AGENT FOR THE APPLICANTS***

12. Name and daytime telephone number of person to contact in the United Kingdom

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# Analytical Method and Apparatus Therefor

The present invention relates to an improved analytical method and apparatus therefor, in particular to a method and apparatus for titration.

Many compounds have physicochemical properties which vary according to their chemical or physical environment, which properties can be investigated by changing that environment and observing the effects on the test compound. Examples of such properties are ionisation state, solubility, partitioning between e.g. organic and aqueous phases or into micelles or liposomes, the strength of ligand binding or metal complexing and hydrophobicity, which can vary with environmental parameters such as pH, ionic strength, or the concentrations of other species in the system. Analytical chemists studying the properties of chemical or biological molecules have long counted titration amongst the major tools of their trade as it allows one parameter of a system, e.g. the pH of a solution, to be varied by dropwise addition of one or more reagents whilst other parameters of the system remain essentially constant, allowing the effects of the variation to be studied effectively in isolation.

An example of a property which can be determined by titration is the pKa (or dissociation constant) of an ionisable group of a compound, which can be defined as the pH at which the group is 50% ionised. The level of ionisation of a given ionisable group at any pH can be directly calculated once the pKa is accurately known. A given molecule may have multiple pKas if it contains more than one ionisable group. As a molecule's state of ionisation can alter other properties such as hydrophobicity and aqueous solubility, knowledge of the pKa(s) of a potential drug molecule is of great importance. To date, because of difficulties with traditional titrimetric techniques, pKa information has not been utilised to the full. Hereinafter, general principles and techniques are discussed in relation to a range of physicochemical properties which may be ascribed to a test compound. Where pKa is discussed, for simplicity it will be assumed that a molecule has only a single ionisable group and therefore a single pKa, however the discussion will apply equally to molecules exhibiting multiple pKas. Where the existence of multiple pKas is of significance this will be addressed specifically.

Traditional titration techniques suffer from many disadvantages. They are slow, at most a small number of samples can be tested per man-day. They are labour intensive, with each dropwise addition of reagent followed by a delay for the mixture to equilibrate before the taking of a reading. The accuracy of conventional techniques is limited by the size of drops added, which can vary with the skill of the operator, and also the concentration of the test compound is altered as each dropwise addition of a reagent dilutes the test sample. Furthermore, relatively large amounts of test compound are required for standard titrimetric techniques, for example if the titrimetric analysis is to determine the pKa of a compound and is monitored by a UV spectrometer then 1mg of test compound may be required. If the same analysis is monitored by a pH meter over 3mg of the compound may be required. Generally spectrophotometric titrations are not automated, whilst potentiometric titrations have been, but even recent attempts at automation have provided slow (1-5 compounds per day), discontinuous techniques and have not removed the need for a skilled laboratory technician to be on hand.

One automated titration system is described by Yarnitzky (Instrumentation Science and Technology, Vol. 23(2), 91-102 (1995) using two peristaltic pumps, a mixing coil and two three-way valves. The system requires the pump drivers to be accurately matched, uses conductimetric or potentiometric detection, and requires compensation for tubing deterioration caused by the pumps, the delay of the mixing coil and the response time of the detector.

In the pharmaceutical industry, as in other branches of chemistry, the current trends towards combinatorial chemistry and recombinant genetic engineering are producing ever more new compounds, ever more quickly. In the pharmaceutical industry, there is a need for the suitability of these new compounds as potential drugs to be evaluated quickly. Several hundred pKa determinations per day may be desirable. The amounts of each compound available for testing may be very small. Consequently there is a need for a more sensitive technique, which can preferably be easily automated for a higher throughput, and which can preferably be operated by laboratory chemists without special training.

Accordingly, the present invention provides a method of continuous titration in which at least one parameter of at least one compound in a test mixture may be monitored as the composition of the mixture is continuously varied. The continuous variation may be characterised by changing concentration of one or more species in the mixture, for example a continuous, preferably linear increase or decrease in the concentration of the species.

In the present method at least two fluid streams are continuously mixed to form a test mixture stream which passes through a spectrophotometric detection zone. The volume to volume ratio of at least two of the component streams in the mixture entering the detection zone is continuously variable with time by alteration of the relative proportions of the component streams forming the test mixture.

In traditional potentiometric and conductimetric titrations the response time of the detector is often the rate limiting step. The use of spectrophotometric detection considerably speeds the titration process, but detection often remains the rate determining step.

The method has the further advantage that, as solutions are not added dropwise but are continuously mixed in varying proportions, the accuracy is no longer limited by the size of drops added. Furthermore, the process can be speeded up considerably; as mixing is continuous, there is no waiting time whilst the mixture equilibrates after addition of each drop. The limiting step is the flow rate achievable through the pumps, mixers and tubes used. A further advantage of the present method is that it can better take advantage of the rate of data sampling at the detector which, in a modern instrument such as a diode array spectrophotometric detector with fixed geometry optics, can be very high e.g. 100 readings per second may be possible although in practical embodiments, 10-30 readings per second, e.g. 20 per second may be taken. High data sampling rates allow the option of "data smoothing" or noise reduction. For example if 20 readings per second are taken, these can be averaged over 10 readings to give an effective sampling rate of 2 per second. This averaging can

provide more sensitive detection than conventional methods of spectrophotometric detection.

Thus, in certain embodiments, the present invention provides a method of continuous titration in which a flowing fluid stream comprising a compound under test is mixed with at least one additional flowing fluid stream to form a test mixture stream and the mixture stream is passed, preferably at a constant flow rate, through a spectrophotometric detection zone at which readings relating to at least one physical or chemical parameter of the compound under test may be taken. Preferably, the test mixture stream is mixed from three fluid components; the first, the volume of which preferably remains constant as a percentage of the total volume of the test mixture, comprises the compound under test. The concentration of this compound in the mixture stream therefore remains constant. The % volumes of the second and third components are preferably variable in inverse proportion to one another; as the % volume of one rises, the % volume of the other falls, so as to keep the total volume of the test sample constant. The variable components may comprise buffer solutions, solvents, test reagents, organic and aqueous phases or other fluid components which may be varied relative to one another to alter the physical or chemical environment of the compound under test. Optionally, further fluid components may be included in the test mixture, at constant or variable volume. For example, salt solutions may be employed to maintain a chosen ionic strength, indicators may be added or the amount of water (or other solvent) may be adjusted to compensate for changes made to the volume of other fluid components.

In especially preferred embodiments, the variable components comprise two linearising buffers - that is two buffers whose relative proportions may be altered to produce a linear pH gradient. These buffers will desirably be formed from components such as an acid and a basic salt of the same compound so that the overall chemical composition of the mixture remains constant during titration and no additional ionic species are introduced. This uniformity of chemical environment gives a measure of predictability to the behaviour of compounds introduced into the titration system, as some compounds behaviour can alter if

the chemical environment changes significantly even, in rare cases leading to the compound precipitating from solution as a solid salt forms.

Thus in one embodiment, in which the pKa of a test compound is to be determined, a test mixture stream is formed from three components: a constant volume of sample solution and two linearising buffer solutions the volumes of which vary in inverse proportion to one another. The absorbance is measured (at a wavelength where there is an absorbance difference between the ionised and unionised forms of the compound) as the proportions of the buffers are varied to produce a linear pH gradient. The pKa of the test compound is the pH at the mid-point of the absorbance change. If the test compound has more than one ionisable group, more than one absorbance change may be observed. The mid-point of the second change then corresponds to the pKa of the second ionisable group.

In a second aspect, the present invention provides an analytical device comprising at least two input ports in fluid communication with a common channel, and a detection zone having an input in fluid communication with the common channel and an output, the device further comprising a spectrophotometric detector for monitoring fluid flowing through the detection zone and producing data relating to at least one chemical or physical characteristic of the fluid. Control means may be associated with the input ports for controlling the relative amounts of fluid introduced into the common channel through each port.

The detector may be any suitable spectrophotometric (i.e. radiation-detecting) analytical detector e.g. an ultraviolet or visible range spectrophotometer, a fluorimeter, a polarimeter, a colourimeter, or a light scattering, optical rotation or circular dichroism detector.

The control means for controlling the relative amounts of fluid introduced into the common channel through each port may be e.g. a pump controller such as is commonly used with HPLC instruments. Alternatively, one or more of the input ports may have associated with it a syringe by which a fluid may be introduced through the port into the common channel, the plungers of the syringes being

moved mechanically under the control of e.g. a computer. The skilled man will be able to envisage other means by which the input of fluids into the common channel may be controlled, such that the proportions of the fluids making up the test mixture and the rate of flow of the test mixture along the common channel through the detection zone may be controlled. The use of pump mixers based on those employed in HPLC instruments, in combination with small-bore tubing and microanalytical detectors in-line, such as fixed geometry optics spectrophotometers, means that very small volumes of test mixture may be used. Consequently, smaller quantities of test compound are needed than were required for traditional titration methods.

In one preferred embodiment, an HPLC mixer pump is connected to reservoirs of each fluid component of the test mixture. The mixer pump takes the fluid containing the test compound at a constant rate and mixes it with a first buffer solution pumped at an increasing rate and a second buffer solution pumped at a decreasing rate, so that the total volume and flow rate of the resulting mixture remains constant, but the relative amounts of each component of the flowing mixture change over time. The changing proportions of the two linearising buffer solutions in the mixture preferably result in changing the pH of the mixture as a whole and are desirably controlled to give a linear pH change over time. Such a system may be used to determine e.g. the pKa(s) of a test compound.

To determine ligand binding test solute is introduced in the manner discussed above, as a constant proportion of the test mixture. Rather than pH being varied as a function time by the mixing of two universal buffers, the ligand of interest is titrated against water or a solvent of relevance in the presence of solute, thus giving a continuous gradient of ligand concentration. The data produced could be analysed using traditional techniques. This approach could be beneficial to other approaches as no dilution factor need be corrected for.

An example of such a system is nickel(II):Ethylenediamine.

In other embodiments, the reservoirs and mixer pump may be replaced by automatic syringes which are controlled e.g. by computer, or by other pumping systems which can handle very small volumes with high precision and accuracy.

Other suitable pumping systems include digital on-off valve pumps in microtubing, and of course the apparatus may comprise two or more different sorts of pump.

The nature and number of fluids mixed to form the test mixture will depend upon the analysis to be performed. For example, if the partition coefficient of a molecule is to be determined, the flow rate into the apparatus of the fluid containing the test compound may be kept constant and those of the two phases between which the molecule will partition may be varied, preferably inversely and linearly. Examples of phase partition fluids which may be employed include oil-in-water emulsions or emulsions of other organic solvents in aqueous solvents (e.g. octanol in water). Surfactant micelles (e.g. sodium dodecyl sulphate (SDS) micelles) and phospholipid, e.g. DPMC liposomes, but the skilled man will be able to select an appropriate mixture to suit the test compound, from his own knowledge. If the parameter to be determined is the binding coefficient of a test compound for a second molecule or other reagent, then the fluids whose proportions are to be varied may include one or both of the binding reagents themselves, and/or salt solutions or buffers for controlling the ionic strength and/or pH of the mixture. Possible interactions which could be studied using the techniques and apparatus of the present invention include those of enzymes for their substrates or for cofactors, chelators for metal ions, receptors for their agonists or antagonists, antibodies for their antigens, or the strength of interaction in any form of complex or specific binding pair. If a compound's solubility in different solvents is being studied, then the levels of two or more different solvents may be adjusted and the effects on the test compound observed. Other examples will readily occur to the skilled man.

As explained above, existing pH meters and conductimetric detectors have particular disadvantages, especially in terms of slow response times. A particular advantage of the present invention is that pH measurement is unnecessary, for example when measuring the pKa of a compound the system may be calibrated over a linear pH gradient between known pH values over a known time period. The start and finish pH values for the gradient can b predicted or measured in advance, or determined from the pKa of the first and last calibration compounds.

Samples of calibration compounds with known pKa values (literature values or values derived by traditional methods) are run against the pH gradient and the gradient time at which the absorbance change mid-point (or of the peak of the 1st derivative of absorbance against time) for each compound is plotted against the literature pKa value for that compound to give a calibration curve.

Compounds of unknown pKa run against the same pH gradient will give a time to peak maximum of the 1st derivative plot, and using this the pKa for that compound can be read from the calibration curve.

The present invention is particularly advantageous in the analysis of poorly soluble compounds, as only very small volumes of solutions are required i.e. 100s of microlitres rather than 100s of millilitres for traditional methods. Also, the use of highly sensitive detectors combined with high data sampling rates allows the introduction of noise-reduction techniques and means that much less concentrated test solutions may be used. Furthermore, it is not necessary to know the concentration of the test compound, because the output can be presented graphically and the changes in e.g. absorbance are plotted rather than absolute values, graphical shape changes showing the changing ionisation state, phase or other changes in the test compound. It is sufficient that the concentration is such that the chromophore is detectable by the spectrophotometer.

Particular embodiments of the present invention are described below, by way of example only, with reference to the accompanying drawings in which:

Fig. 1 is a diagrammatic representation of apparatus according to a first embodiment of the invention;

Fig. 2 is a diagrammatic representation of apparatus according to a second embodiment of the invention;

Fig. 3 is a diagrammatic representation of the plumbing connections of the apparatus of Fig 2;

Fig. 4 is a diagrammatic representation of the electrical connections of the apparatus of Fig 2;

Fig. 5 is a diagrammatic representation of the electrical trigger events controlling the apparatus of Fig 2;

Fig. 6 is a detailed diagram showing the connections within the terminal block of the apparatus of Fig 2;

Fig. 7 is a diagrammatic representation of apparatus according to a third embodiment of the invention;

Fig. 8 is a diagrammatic representation of the relationship between pKa, absorbance and 1st derivative of absorbance for a species having a single ionisable group in which the ionised and unionised forms have different absorbance profiles;

Fig. 9 shows a standards (calibration) curve derived from titration data obtained in accordance with the invention for compounds of known pKa;

Fig. 10 is a plot of pH against time for the linear gradient.

Fig. 11 is an absorbance curve for 4-CN phenol run on the gradient of Fig. 10;

Fig. 12 is a plot of the first derivative of the absorbance readings plotted in Fig. 11;

Fig. 13 is an absorbance curve for an endpoint titration; and

Fig. 14 is a calibration curve for an endpoint titration.

Fig. 15 is a plot of pH against % acid for the linear gradient on which the standards (calibration) curve of fig 9 was produced.

Examples

1. Apparatus

The apparatus has been assembled from equipment already available in the laboratory, and consists of the following units:

Gilson Aspec XL autosampler;
Hewlett Packard 1050 quaternary HPLC pump;
Kontron 440 diode array detector (spectrophotometric detector);
66 MHz 486 PC computer with Strawberry Tree Data acquisition card;
Dynares 8 Ultra (+71-TC) terminal panel;
Dasylab software is used for data capture.

Diagrammatic representations of two arrangements of the apparatus can be seen in figures 1 and 2. The plumbing connections between the various units in the figure 2 arrangement are shown in figure 4 and the electrical connections in Figure 5.

Purpose-built equipment comprising a sampler or other sample reservoir and delivery device, or pump/mixer assembly, a spectrophotometric detector and a data processor can be envisaged and will be apparent to persons skilled in the art who will be able to put them into operation without undue burden.

Improvements to Buffering System

The system initially developed used four solutions mixed into a linear gradient (Fig. 1). The sample at constant % volume was titrated with acid (as in Fig. 1) or base and the % volume of salt solution was decreased as the acid or base increased, to maintain ionic strength within acceptable limits. The system was buffered by a constant % volume of buffer solution. Refinement of the buffering system has allowed this to be reduced to three components; a sample solution, the amount of which is not varied over the time that the gradient is run, and two linearising buffer solutions, one acidic and one basic, which are varied linearly over time in inverse proportion to one another. See Fig. 2.

Operation Of The Apparatus

During the running of the gradient, the sample containing the test compound is drawn at a constant rate from the autosampler into channel A of the HP1050 pump. At the same time, varying amounts of the other components are drawn into the pump. Universal buffer component B (basic component, see further below) is drawn into channel B from a reservoir. Similarly universal buffer compound A (acidic component) is drawn into channel C. One of the buffer components rises from zero or a low % volume of the test mixture at the start of the gradient to e.g. 80% or more of the mixture at the end. The other buffer falls from e.g. 80% or more of the mixture to zero or a low final concentration. For a gradient of increasing pH, the proportion of buffer B will rise over the time of the gradient whilst the proportion of buffer A will fall. The remainder of the mixture is test compound solution, optionally with other components as necessary (e.g.

water, surfactant micelles, reactant(s)) which may be supplied via channel D of the HPLC pump.

The mixed components pass from the outlet of the HPLC pump to the Spectrophotometer (Kontron 440DAD) and then to waste, optionally via a pH meter which may be used to monitor the correct operation of the system, e.g. to check the linearity of the pH gradient formed.

Tubing may suitably be 1/16" OD PEEK or stainless steel tubing.

When a number of samples are to be tested, the apparatus can be set to run through a repeating cycle in which the first test sample, the buffers and any other components are at first pumped through the HPLC pump at constant rates in fixed ratios. This gives a stable starting point for the gradient. The gradient is then run by varying the ratios of the buffer components, and the final conditions of the gradient may then be maintained for a short period before the system recycles (which may include flushing with water of other suitable solvent at the end of the cycle), in preparation for the drawing up of the next sample.

Electrical Connections

As can be seen from the outline of the electrical connections depicted in figure 4, analog data from the spectrophotometer and pH meter are fed to the terminal block and thence to the PC for capture and analysis by Dasylab software. Any other software capable of capturing and manipulating analog data would be suitable. This embodiment is limited to four analog outputs from the spectrophotometer, the four data channels from the spectrophotometer are connected to shielded inputs 1 to 4 on the terminal block and the analog signal from the pH meter is connected to terminal 5. Shielding of the cables reduces interference from high frequency instrumental noise. The connections within the terminal block are shown in more detail in figure 6.

As can be seen from figure 4, the autosampler is connected to the pump, spectrophotometer and terminal block. These contacts are digital signals which specify the start and finish of the experimental cycle, these contact closure events are driven by the autosampler. The signals to the pump and

spectrophotometer are contact closures, the signal to the terminal block is a contact opening.This is shown in more detail in figure 5.

Figure 7 shows a similar apparatus arrangement to that of figure 2, but the buffer components are introduced into the mixer from automatic syringes rather than being drawn up by the mixer pump from a reservoir. Any extra components such as micelle suspension for a partitioning experiment may also be introduced by syringe as may the test samples, if desired, although if multiple samples are to be tested the autosampler provides a convenient means of automation.

2. Determination of pKas

The method of continuous titration permits the creation of a fast linear pH gradient over a wide pH range, with the use of appropriate buffers as described below. This in turn allows the speedy determination of pKa values.

Where a molecule has a single ionisable group and the ionised and unionised forms have different UV absorbance spectra, an absorption change will be detected as the mixture of ionised and unionised forms changes from predominantly ionised to predominantly unionised (or *vice versa*). See the generalised diagrammatic representation of Fig. 8 and, for a practical example, Figs. 11 and 12. For such a single ionisable group, the pH corresponding to the mid-point of the change in absorbance is the pKa of the compound (the pH at which 50% of the molecules are ionised). The mid-point of this curve can be determined by curve-fitting or, preferably, by taking the 1st derivative of the absorbance readings against pH. This gives a peak, the maximum of which marks the mid-point of the absorbance curve and thus the pKa. Use of the first derivative plot allows pKas which lie close to the ends of the pH gradient to be determined, as the gradient need only run a short way past the pKa for the first derivative plot to peak and begin its down-turn. By contrast, the mid-point of the absorbance trace can only easily be determined if the lowest and highest absorbance levels can be seen on the trace, which requires a longer span of the pH gradient, as can be seen from figure 8.

The apparatus is fitted with a pH meter so that absorbance can be determined over time or against pH. However, if the speed of the pH gradient is too fast, the pH electrode may not be able to respond quickly enough, giving erroneous readings. In this situation, the gradient can instead be calibrated using compounds of known pKa. Linear regression of the known pKas of standard compounds against the time of the peak maximum in the 1st derivative of the absorbance curve for that compound yields a calibration curve which can be used to determine the pKa of unknown compounds without pH measurement. The "time to peak maximum" of a test compound run through the same gradient is determined and the pKa is read off from the calibration curve. This "time to peak maximum" may be measured from the start of the apparatus cycle or from the start of the gradient, as convenient, the important criterion being that a consistent start time is used for all standard and test compounds run on the same gradient. Although calibration of the gradient is being used instead of direct pH measurement, the pH electrode may be kept in place as a diagnostic tool, e.g. to check correct instrumental operation, such as whether the pH gradient remains linear.

Where a molecule has more than one ionisable group, more than one peak may be observed in the first derivative trace and the two or more pKas may be individually read from the calibration curve, corresponding to the different peak times. Overlapping pKa's will require further mathematical treatment.

Figure 2 shows a diagrammatic representation of apparatus used to form a buffered linear pH gradient. A Gilson Aspec autosampler was used with a HP1050 quaternary HPLC pump with a Kontron model 440 diode-array detector and an in-line Pharmacia flow-through pH electrode and meter. Data from the detector and pH meter were collected on computer.

A linear pH gradient was created by mixing a sample solution, the amount of which is not varied over the time that the gradient is run, and two buffer solutions, one acidic and one basic, which are varied linearly over time in inverse proportion to one another. The two buffers have a common component to which an acidic component is added to form buffer A and a basic component is added to form buffer B. The buffers were made up as follows:

Solution C: Common Component (1 litre)

Into 1 litre of water:

| | | | |
|---|---|---|---|
| Boric Acid (FLUKA 15660) | 24.732g | (Mw 61.83) | =0.4M |
| TRIS (FLUKA 93350) (hydroxymethyl)-aminomethane) | 48.456g | (Mw: 121.4) | =0.4M |
| Butylamine (FLUKA 19480) | 29.256g (Mw 73.14, density 0.737) | (39.696 $cm^3$) | =0.4M |

Buffer A - 1 litre

Into 500 $cm^3$ Solution C:

| | | |
|---|---|---|
| $KH_2PO_4$ (BDH ANALAR 10203) | 27.218g | (Mw 136.09) |
| Citric Acid Monohydrate | 42.028g | (Mw: 210.14) |
| HCl | 350 $cm^3$ | (1m solution) |

made up to 1000 $cm^3$ total volume with $H_2O$ to give:

| | |
|---|---|
| $KH_2PO_4$ | 0.2M |
| Citric Acid Monohydrate | 0.2M |
| HCl | 0.35M |
| pH = | ~2.8 |

Buffer B - 1 litre

Into 500 $cm^3$ Solution C:

| | | |
|---|---|---|
| $K_2HPO_4$ (FLUKA 60356) | 34.836g | (Mw: 174.18) |
| $K_3$Citrate (Monohydrate) (FLUKA 60153) | 64.884g | (Mw: 324.42) |
| KOH (ALDRICH 31,936-8) | 400 $cm^3$ (0.5M) | |

made up to 1000 $cm^3$ total volume with $H_2O$ to give:

| | |
|---|---|
| $K_2KPO_4$ | 0.2M |
| $K_3$Citrate | 0.2M |

KOH 0.2M

pH = ~ 11.58

Buffer component A (acidic) and B (basic) need to be diluted 1:10 before use in an HPLC gradient.

This gives pH values of the diluted buffers as follows:

Acidic (Buffer A) = 3.01
Basic (Buffer B) = 11.19

The linearity of this buffer system was tested stepwise by using an HP1050 HPLC pump to mix the buffers at a flow rate of $5cm^3$ $min^{-1}$, with the pH being monitored with a flow-through Pharmacia pH electrode. The relative amounts of buffer A and buffer B were kept constant until the pH reading was stable then stepped to their next values and held again until a stable reading was achieved before being stepped once more. This was repeated until the gradient was completed.

The results are set out below and represented graphically in Fig. 15

| % Buffer A | % Buffer B | pH |
|---|---|---|
| 100 | 0 | 2.91 |
| 90 | 10 | 3.9 |
| 80 | 20 | 4.71 |
| 70 | 30 | 5.45 |
| 60 | 40 | 6.2 |
| 50 | 50 | 6.98 |
| 40 | 60 | 7.77 |
| 30 | 70 | 8.57 |
| 20 | 80 | 9.46 |
| 10 | 90 | 10.39 |
| 0 | 100 | 11 |

Figure 15 shows that the pH gradient is essentially linear from pH 3 to 11.

Compounds of known pKa were run in a continuous (rather than stepped) gradient in which the amount of buffer A ran from 80% to 0% and of buffer B from 0% to 80% of the test mixture over 4 minutes. The sample solution was kept constant at 20%.

The HP1050 pump was used again with buffer B introduced via channel B and buffer A via channel C. The flow rate of the test mixture stream from the mixer to the detector was 1 $cm^3$ $min^{-1}$.

The absorbance changes at 240 nm, 265 nm, 290 nm and 315 nm were recorded and the peak maxima of the 1st derivative plots determined. A calibration curve (Fig. 9) was created from the time to peak maxima and the known pKa values of the standards. Compounds of known pKa were also determined as test solutes.

The calibration results are set out below: Time to peak maximum (RT) is from the start of the instrument cycle (when the autosampler first goes into a new sample container).

Standards

| | **pKa*** | **Time to peak maximum** | | |
|---|---|---|---|---|
| Benzoic acid | 3.96 | 217.3 | | |
| Phenol | 9.766 | 444.8 | | |
| phthalate 1 † | 4.82 | 251.8 | | |
| 4-$NO_2$phenol | 6.89 | 334.7 | Intercept | -1.5717 |
| Benzoic acid | 3.96 | 216.3 | Slope | 0.0254 |
| Phenol | 9.766 | 445 | | |
| phthalate 1 | 4.82 | 249.8 | R2= | 0.9998 |
| 4-$NO_2$phenol | 6.89 | 334.9 | | |
| Benzoic acid | 3.96 | 216.5 | | |
| Phenol | 9.766 | 444.3 | | |
| phthalate 1 | 4.82 | 251.5 | | |

| | | |
|---|---|---|
| 4-$NO_2$phenol | 6.89 | 334.7 |

† the more alkaline of the two potassium hydrogen phthalate pKa values.

* determined by potentiometric titration on Sirius PCA 101 instrument in 0.15 KCl.

Results for the test solutes and residuals are set out below:

| Sample | Known pKa* | Time to Peak Maximum | pKa Derived | Residual |
|---|---|---|---|---|
| 3-Cl phenol | 8.81 | 407.5 | 8.79 | 0.02 |
| 4-Cl phenol | 9.14 | 421.2 | 9.14 | 0.00 |
| 2-Cl phenol | 8.24 | 382.8 | 8.16 | 0.08 |
| 4-CN phenol | 7.7 | 360 | 7.58 | 0.12 |
| 3-Cl phenol | 8.81 | 406.8 | 8.78 | 0.03 |
| 4-Cl phenol | 9.14 | 420.8 | 9.13 | 0.01 |
| 2-Cl phenol | 8.24 | 382.3 | 8.15 | 0.09 |
| 4-CN phenol | 7.7 | 361.3 | 7.62 | 0.08 |
| 3-Cl phenol | 8.81 | 407.3 | 8.79 | 0.02 |
| 4-Cl phenol | 9.14 | 421 | 9.14 | 0.00 |
| 2-Cl phenol | 8.24 | 382.3 | 8.15 | 0.09 |
| 4-CN phenol | 7.7 | 360 | 7.58 | 0.12 |

* determined by potentiometric titration on Sirius PCA 101 instrument in 0.15 KCl.

The derived pKa values taken from the standards calibration curve are very close to those expected.

Figs. 10,11 and 12 show the calibration curve (Fig. 10) with the absorbance curve at 290 nm (Fig. 11) and the 1st derivative plot (Fig. 12) for 4-CN phenol run on the above gradient. When the pKa value corresponding to the time of the peak maximum (361 seconds) is read from the calibration curve for this pH gradient, the pKa derived is 7.64. The expected result is 7.7 (derived from traditional stepwise titration using the Sirius PCA101 instrument).

The method could be further enhanced by incorporation of the calibration curve into the data handling routines, for example the computer which stores the absorbance readings generated by the detector may be programmed to find the first derivatives of these readings, determine the time of the peak reading and, for example using a look-up table derived from the calibration curve absorbance readings, produce an output reading giving the pKa of the sample. The pKa reading would then be the only output - no calculations would be required on the part of the operator.

3. Determination of Partitioning Into Micelles

An important attribute of certain drug molecules is that they may partition across certain barriers, such as phospholipid membranes. Generally, one of the ionisation states of a given molecule will cross the barrier more efficiently than the other(s). At a given pH, a certain constant proportion of the molecules in free solution will be ionised, but individual molecules will be switching between ionised and unionised states: it is a dynamic equilibrium. If, for example, the unionised form has the greater tendency to partition into the micelles, then the addition of micelles causes the concentration of that species in free solution to drop, as the molecules cross into the micelles. The dynamic equilibrium between the ionised and unionised forms in the free solution adjusts to this, by a drop in the concentration of the ionised species and a rise in the concentration of the unionised species, until the initial equilibrium ratio is re-established.Thus the observed absorbance mid-point (apparent pKa) is shifted when a pH gradient is run in the presence of micelles. This results in an observed shift in the pKa of the compound, increasing for acids, decreasing for bases.The log P of the compound can be derived directly from this shift in apparent pKa and a knowledge of the volume ratios of the two phases. One assumption of this approach is that the absorption characteristics of the molecules do not change significantly between phases.

This behaviour can be studied using the continuous titration method and apparatus described above by including a fourth component in the gradient mixture. This component comprises micelles formed from surfactants such as sodium dodecyl sulphate (SDS). The concentration of surfactant in the fourth component must be high enough that in the final test stream, mixed from the four components, the

surfactant is present in excess of its critical micelle concentration (CMC) and micelles are formed.

To determine partitioning, the amounts of the micelles suspension and of the sample solution are maintained constant as the pH gradient is run. The partitioning coefficient can be determined by the following equation:

$$\log P = \log (\Delta pKa\ (Vw/Vo))$$

where ΔpKa is the difference in pKa in the presence and absence of micelles, Vw is the volume of the aqueous phase and Vo is the volume of the organic phase (micelles). Vo can be calculated from the CMC, micelle radius and the aggregation number of the surfactant (number of molecules required for each micelle), factors which would be readily available to or calculable by the skilled man.

Partitioning of Benzoic Acid into SDS Micelles

This experiment was run to observe partitioning into micelles using continuous titration. The continuous titration apparatus was set up as in Example 1 (Fig. 2).

Four concentrations of SDS were used, derived from a 0.1M stock solution in water.

The following samples were prepared, in a total volume of 20ml.

| Sample | Volume SDS Stock | Volume Benzoic Acid Stock | Volume $H_2O$ | Conc. SDS (M) | Conc. Benzoic Acid (mM) |
|---|---|---|---|---|---|
| A | 0 | 20 | 0 | 0 | 0.77 |
| B | 20 | 0 | 0 | 0.1 | 0 |
| C | 20 | 0* | 0 | 0.1 | ~0.7 |
| D | 15 | 0 | 5 | 0.075 | 0 |
| E | 15 | 5 | 0 | 0.075 | 0.19 |
| F | 10 | 0 | 10 | 0.05 | 0 |
| G | 10 | 10 | 0 | 0.05 | 0.39 |
| H | 5 | 0 | 15 | 0.025 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| I | 5 | 15 | 0 | 0.025 | 0.58 |

* ~0.5 mg solid benzoic acid

The continuous titration apparatus was set up with the following sample queue:

1. Blank (water only)
2. Blank (water only)
3. Blank (water only)
4. STD1
5. STD2
6. A
7. B
8. C
9. D
10. E
11. F
12. G
13. H
14. I
15. STD1
16. STD2
17. Blank (water only)
18. Blank (water only)

The vials containing no benzoic acid (SDS blanks) did not show any titration curves and so have been omitted from any further handling (vials 7, 9, 11 and 13).

The results are summarised below:

**Standard Curve**

| Vial | | Compound | pKa | RT |
|---|---|---|---|---|
| 4 | STD1 | KHP | 4.878 | 299.5 |
| | | Phenol | 9.721 | 447.8 |
| 5 | STD2 | Benzoic acid | 3.964 | 272 |
| | | p-$NO_2$ phenol | 6.869 | 363.8 |
| 15 | STD1 | KHP | 4.878 | 300.5 |
| | | Phenol | 9.721 | 448.5 |
| 16 | STD2 | Benzoic acid | 3.964 | 272.5 |
| | | p-$NO_2$ phenol | 6.869 | 363.5 |

Intercept = -4.9325

Slope = 0.0326

**Samples**

| Vial | Compound (conc. SDS) | Time to Peak | pKa1 | Delta pKa |
|---|---|---|---|---|
| 6 | Benzoic acid (0.0M) | 271 | 3.91 | |
| 8 | Benzoic acid (0.02M) | 275.8 | 4.07 | 0.16 |
| 10 | Benzoic acid (0.015M) | 275.8 | 4.07 | 0.16 |
| 12 | Benzoic acid (0.01M) | 274.5 | 4.02 | 0.11 |
| 14 | Benzoic acid (0.005M) | 274 | 4.01 | 0.10 |

Calculation of Log P

$$\log P = \log (\Delta pKa \times {}^{Vw}/_{Vo})$$

where Vw = volume of aqueous phase

Vo = volume of organic phase

For this exercise Vo is taken as the volume of SDS micelles.

At 0.1M ionic strength (the ionic strength of the buffer stream)
Micelle radius = $2.5 \times 10^{-9}$m
Aggregate number - ~100
Critical Micelle Concentration: 1.5 mM.
(From Van Os N.M. et.al: Physico-chemical properties of selected Anionic,Cationic and nonionic surfactants. Elsevier ISBN:0-444-89691-0).

All surfactant present above the CMC is present in the form of micelles, so from the above information we can calculate the volume of SDS micelles present in each solution.

Volume of Sphere

$$V = \frac{4\pi r^3}{3} = 6.54 \times 10^{-26} M^3$$

| Sample | pKa | Final SDS conc. (Molar) | SDS excess (Molar) | SDS molecules | SDS micelles | SDS volume (ml) | Vol Ratio | Delta pKa | P | log P |
|---|---|---|---|---|---|---|---|---|---|---|
| Benzoic acid | 3.91 | | | | | | | | | |
| Benzoic acid in 0.1M SDS | 4.07 | 0.02 | 0.0185 | $1.11 \times 10^{22}$ | $1.11 \times 10^{20}$ | 0.00729 | 137.25 | 0.16 | 21.96 | 1.34 |
| Benzoic acid in 0.075M SDS | 4.07 | 0.015 | 0.0135 | $8.13 \times 10^{21}$ | $8.13 \times 10^{19}$ | 0.00532 | 188.08 | 0.16 | 30.09 | 1.48 |
| Benzoic acid in 0.05M SDS | 4.02 | 0.01 | 0.0085 | $5.12 \times 10^{21}$ | $5.12 \times 10^{19}$ | 0.00335 | 298.72 | 0.11 | 32.86 | 1.52 |
| Benzoic acid in 0.025M SDS | 4.01 | 0.005 | 0.0035 | $2.11 \times 10^{21}$ | $2.11 \times 10^{19}$ | 0.00138 | 725.46 | 0.1 | 72.55 | 1.86 |

Average log P = 1.55
Stdev 0.11

The experiment appears to have worked very well, a consistent shift in pKa was observed, which gives reasonable results in all SDS concentrations.

These results indicate that continuous titrations can be used for measuring partitioning into organised organic phases such as micelles.

4. End Point Titrations

Many traditional quantitative titration techniques, for example determination of the concentration of a compound in a solution, rely upon the use of a visual end-point indicator. The accuracy of such techniques are heavily reliant upon the skill of the operator and visual interpretation of the endpoint indicator. Using continuous gradient titration with spectroscopic detection of endpoints will make the accuracy of the technique independent of operator skills. The technique will be especially applicable to compounds with a single ionisable group or a small number of non-overlapping pKas.

An example of the use of this technique is the determination of potassium hydrogen phthalate (KHP) concentration by endpoint determination using phenolphthalein indicator.

The test solute (KHP) was introduced in the sample stream at a constant 20% of the final mixture, as in Example 1 above. The two components of the gradient (from 80% to zero and zero to 80% respectively) are 0.05M KOH and water. The end-point indicator was introduced via the sample stream (2 drops phenolphthalein solution in 20cm$^3$ of sample).

The test solute KHP was titrated by the KOH stream. As soon as all compound has been titrated there is a large increase in pH and rapid change of the ionisation state of the indicator and hence a rapid colour change. The system was calibrated by the use of KHP standards of known concentration.

Chemicals

0.05M KOH - channel B

$50cm^3$ 0.5-KOH (Aldrich) diluted to $500cm^3$ in $H_2O$

0.27g phenolphthalein indicator weighed out and dissolved in $10cm^3$ MeOH and $10cm^3$ $H_2O$. Some precipitation did occur.

KHP solution 0.1416M

A series of dilutions were made to yield the following KHP solutions

0.1416M
0.0708M
0.0354M
0.0177M
0.0089M

These standards were decanted into scintillation vials and 2 drops of indicator added. The samples and a blank were then run. Absorbance was measured at 240nm.

Example traces obtained are shown in Figure 13.

The peak times obtained for the blank and standards were entered into an Excel spreadsheet and a regression of KHP concentration against gradient time performed.

| **Determination of KHP by Continuous Gradient Titration** | |
|---|---|
| **Conc. KHP (M)** | **Peak Time** |
| 0.1416 | 398 |
| 0.0708 | 292 |
| 0.0354 | 241 |
| 0.0177 | 216.5 |
| 0.0089 | 203.5 |
| | |
| 0 | 186.5 |

A very good regression was obtained with highly significant statistics $r^2 = 0.9996$ F=10337 - the calibration curve plotted is shown in Fig. 14. Unknown concentrations of KHP run on the same gradient can be determined from this 1st derivative peak absorbance time using this calibration curve.

This experiment has shown the applicability of continuous gradient titration to classical end-point titrations.

This approach should have several benefits over traditional approaches.

1. Fast, high throughput;
2. Very sharp end-point detection, high accuracy;
3. No user knowledge required;
4. Large dynamic range.

It will be apparent to the skilled man that variations of the above are possible, for example instead of calibrating the system using several standards run before or between the samples, the standard compounds of known pKa could be included in the sample solution to provide internal standards. These would give absorbance change times for known pKas against which the test compound could be compared. Other adaptations would be apparent to the skilled man which may be put into practice with the aid of standard laboratory techniques and without undue burden.

The continuous titration apparatus described above may be further automated by the use of e.g. autosamplers and appropriate computer software. For example, a system can be envisaged in which dilute solutions of compounds for testing are prepared by the chemist and placed in an autosampler rack. The pKa measurement and a printed report would be produced automatically by the computer receiving data from the spectrophotometer. Alternatively, the system could be interfaced to a screening robot by which aliquots of compounds in test solutions are dispensed, diluted and injected into the continuous titrator. After titration the pKa results could be printed automatically and/or written to a database.

The application of which this description and claims forms part may be used as a basis for priority in respect of any subsequent application. The claims of such subsequent application may be directed to any feature or combination of features described herein. They may take the form of product, composition, process or use claims and may include, by way of example and without limitation, one or more of the following claims:

Claims:

1. A method of continuous titration in which at least one parameter of at least one compound in a test mixture may be monitored as the composition of the mixture is continuously varied by changing concentration of one or more species in the mixture, the method comprising the steps of continuously mixing at least two component fluid streams to form a test mixture stream and passing the test mixture stream through a spectrophotometric detection zone, characterised in that the volume to volume ratio of at least two of the component streams forming the test mixture stream is continuously varied with time by alteration of the relative proportions of the component streams forming the test mixture.

2. A method according to claim 1 wherein the test mixture stream is formed from three component fluid streams, the proportion of one component fluid stream remaining constant, the proportions of the second and third component fluid streams being variable in inverse proportion to one another.

3. A method of continuous titration comprising mixing a flowing fluid stream comprising a compound under test with at least one additional flowing fluid stream to form a test mixture stream and passing the test mixture stream, at a constant flow rate, through a spectrophotometric detection zone at which readings relating to at least one physical or chemical parameter of the compound under test are taken.

4. A method according to claim 2 wherein the second and third component fluid streams comprise two linearising buffer solutions.

5. An analytical device comprising at least two input ports in fluid communication with a common channel and a detection zone having an input in fluid communication with the common channel and an output, the device further comprising a spectrophotometric detector for monitoring fluid flowing through the detection zone and producing data relating to at least one chemical or physical characteristic of the fluid.

6. An analytical device according to claim 5 further comprising control means associated with the input ports for controlling the relative amounts of fluid introduced into the common channel through each port.

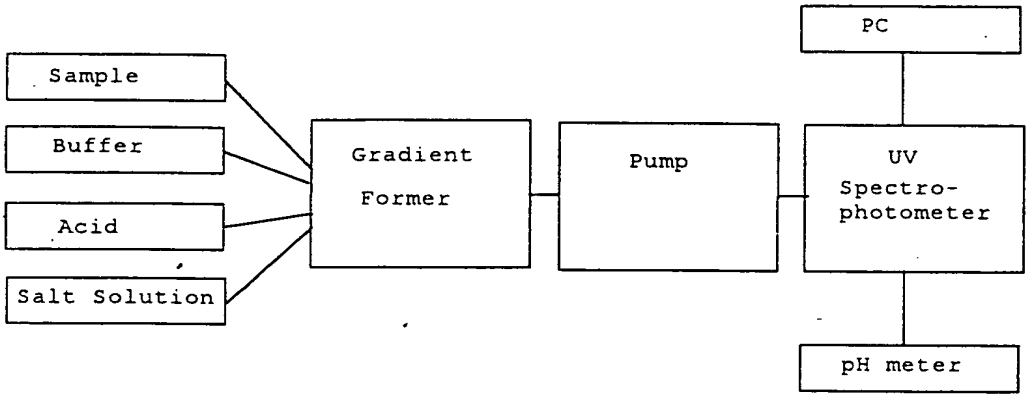
PC
Sample
Buffer
Acid
Salt Solution
Gradient
Former
Pump
UV
Spectro-
photometer
pH meter

Fig. 1

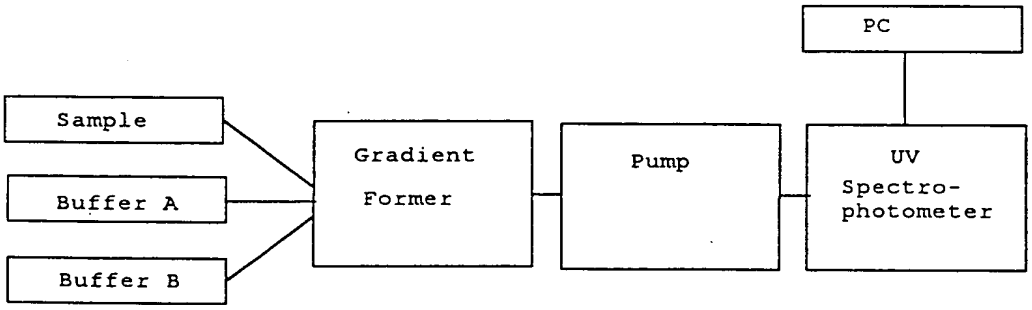
PC
Sample
Buffer A
Buffer B
Gradient
Former
Pump
UV
Spectro-
photometer

Fig. 2

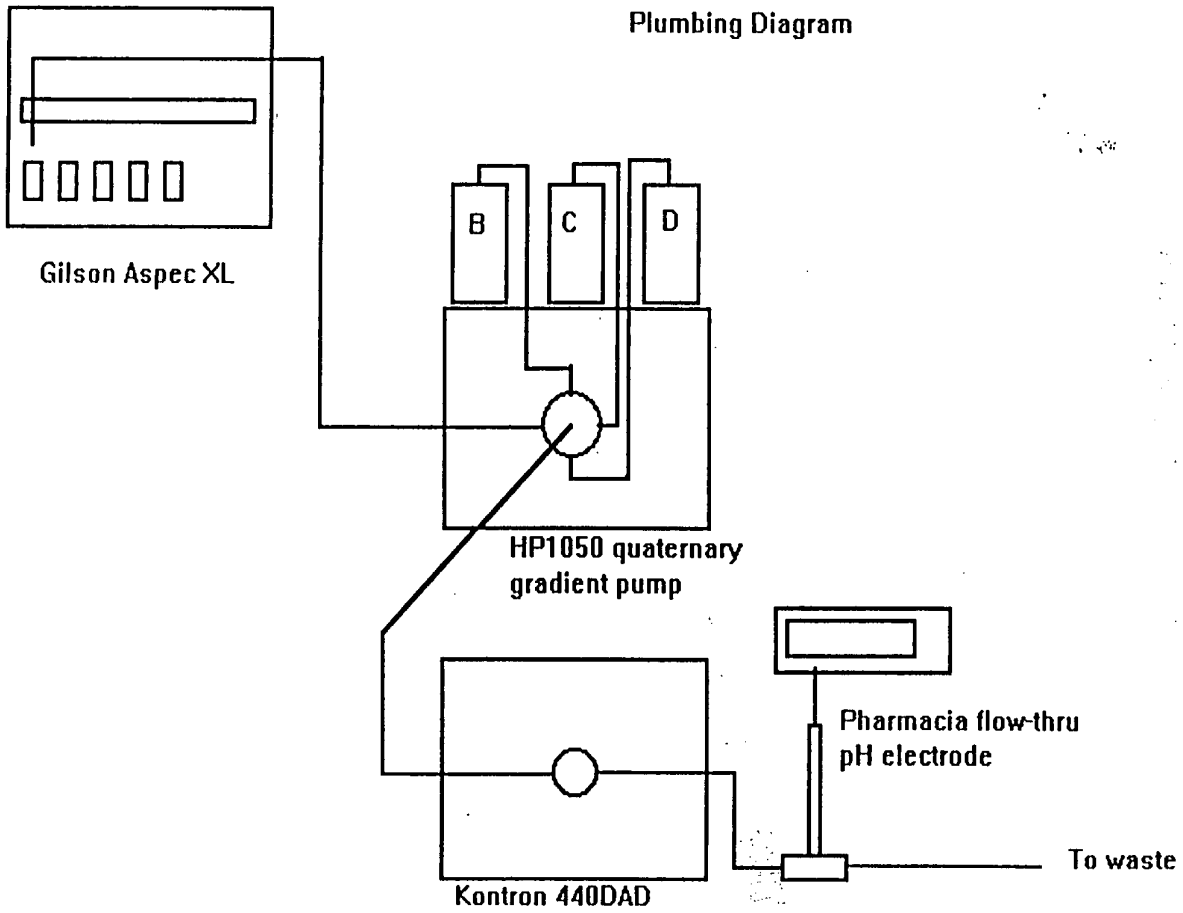
Plumbing Diagram
Gilson Aspec XL
B
C
D
HP1050 quaternary
gradient pump
Pharmacia flow-thru
pH electrode
To waste
Kontron 440DAD

FIG. 3

electrical connections 1 : Outline

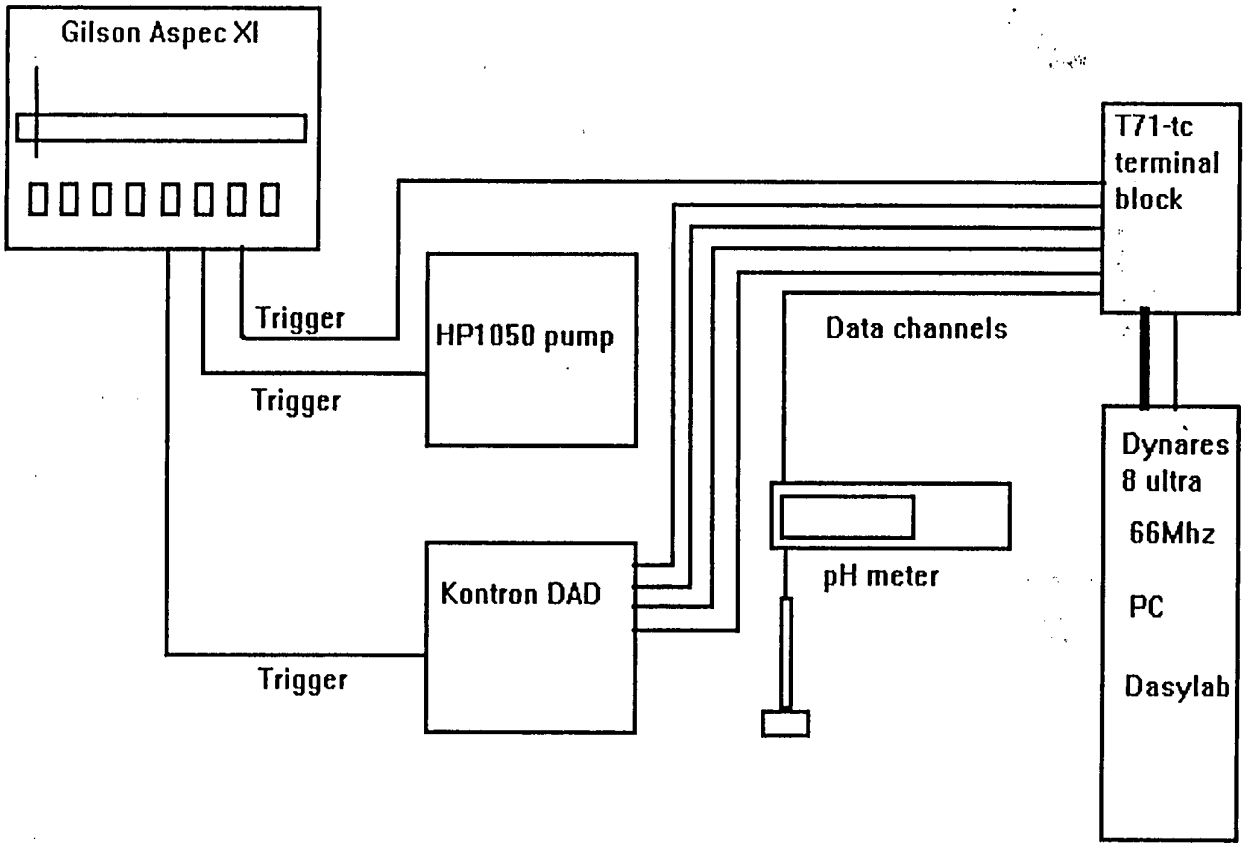


FIG. 4

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electrical connections 2: Trigger events

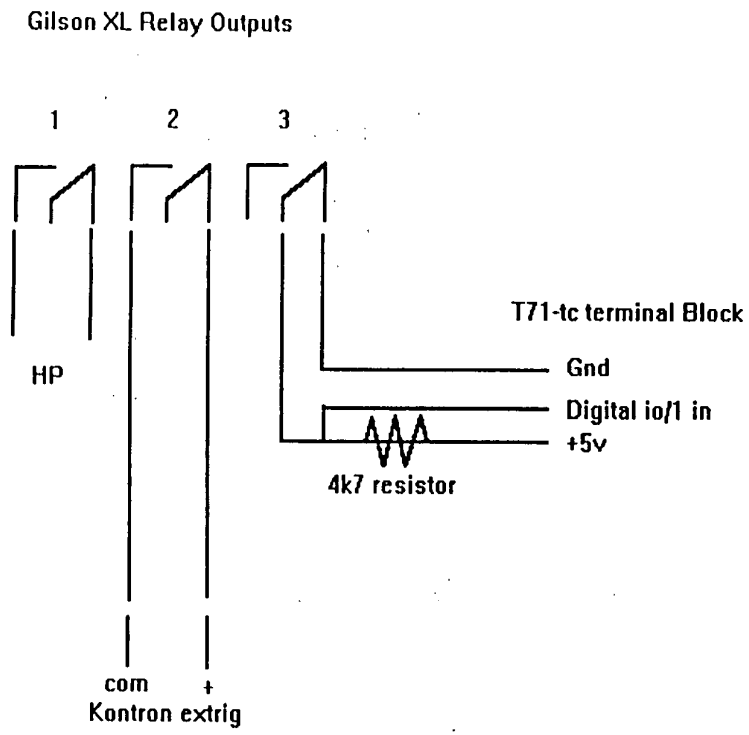



FIG. 5

electrical connections 3: T71-tc terminal block

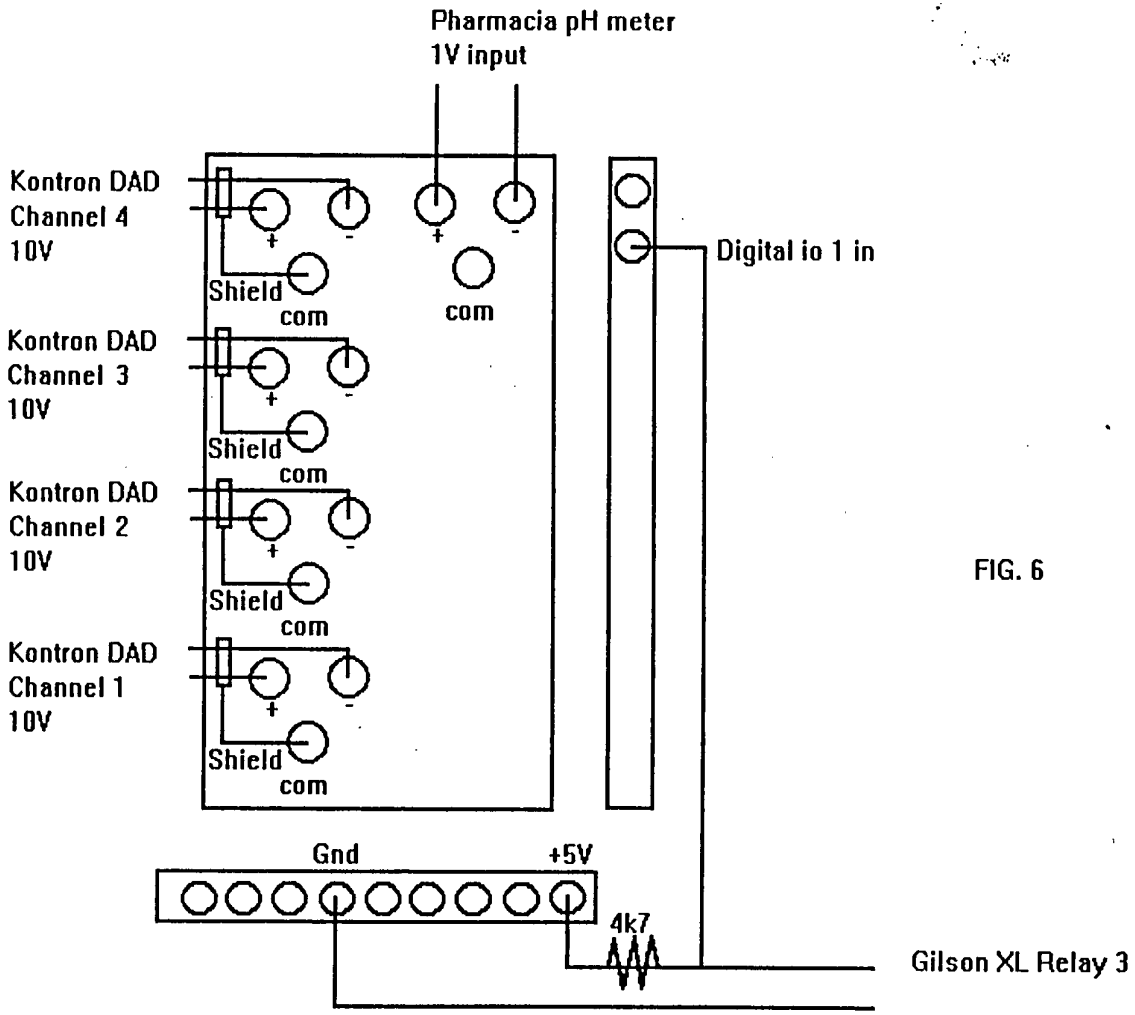



FIG. 6

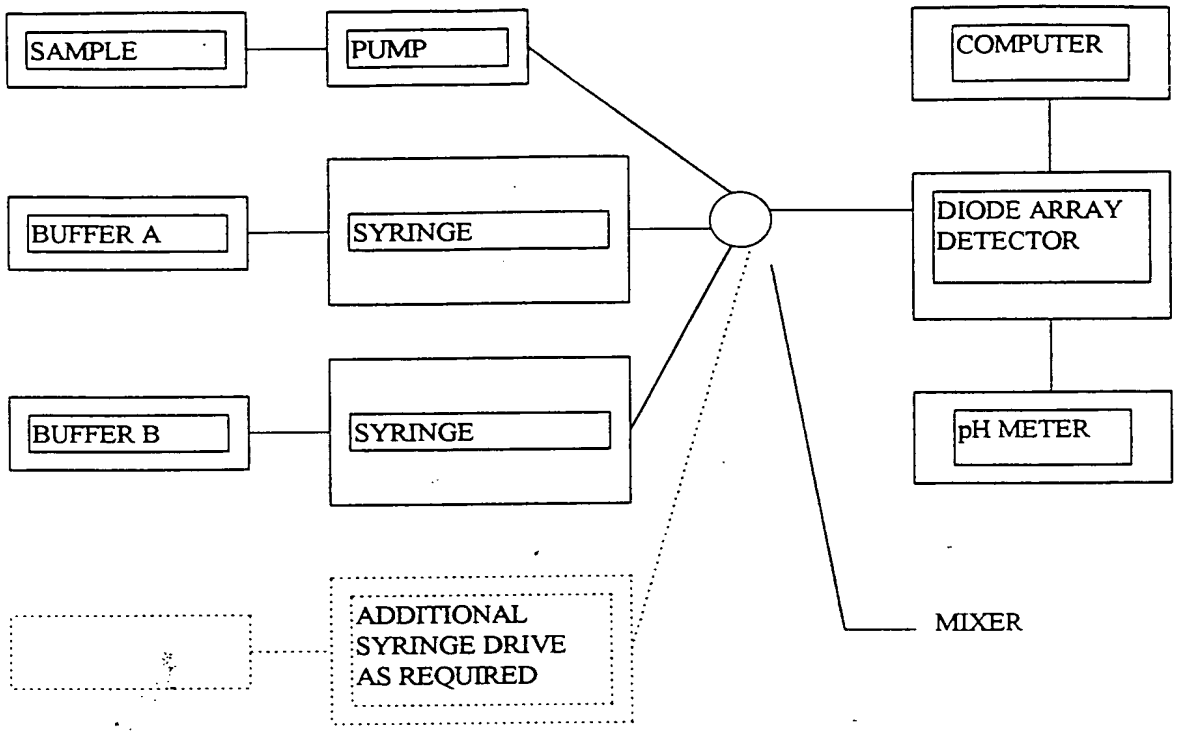

SAMPLE
PUMP
COMPUTER
BUFFER A
SYRINGE
DIODE ARRAY DETECTOR
BUFFER B
SYRINGE
pH METER
ADDITIONAL SYRINGE DRIVE AS REQUIRED
MIXER

Fig. 7.

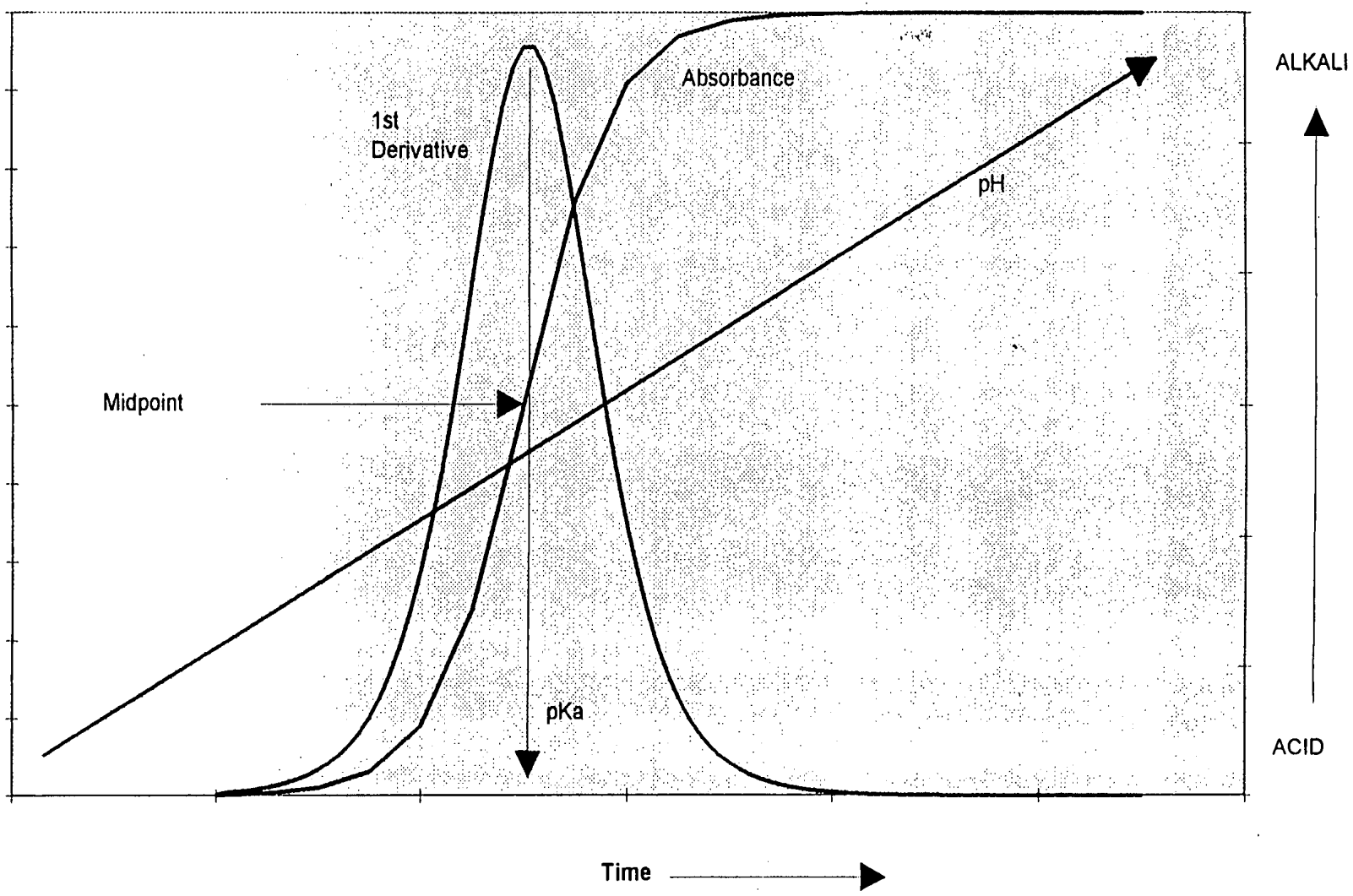

Absorbance
1st
Derivative
pH
ALKALI
Midpoint
pKa
ACID
Time

Fig. 8

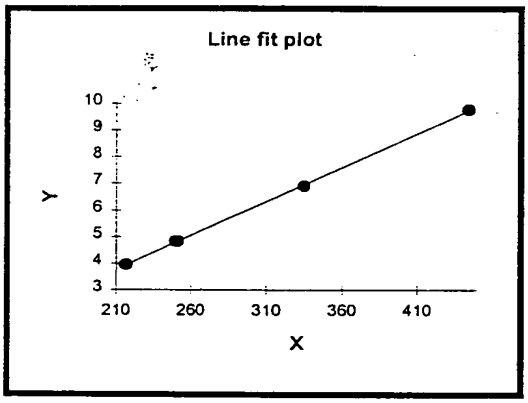
Figure 9: pKa standards calibration curve

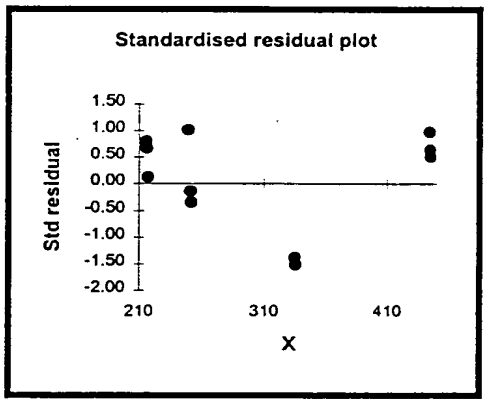
**Linear regression** PGP pKa calibration

| | |
|---|---|
| n: | 12 |
| R Square: | 0.9998 |

| | Coefficient | SE | *t* | *p* | 95% CI of Coefficient |
|---|---|---|---|---|---|
| Intercept: | -1.5717 | 0.0398 | -39.5215 | <0.0001 | -1.6603 to -1.4831 |
| Slope: | 0.0254 | 0.0001 | 207.1994 | <0.0001 | 0.0252 to 0.0257 |

| | Sum of Squares | *df* | Mean Square | *F* | *p* |
|---|---|---|---|---|---|
| Regression: | 60.026 | 1 | 60.026 | 42931.5774 | <0.0001 |
| Residual: | 0.014 | 10 | 0.001 | | |
| Total: | 60.040 | 11 | | | |

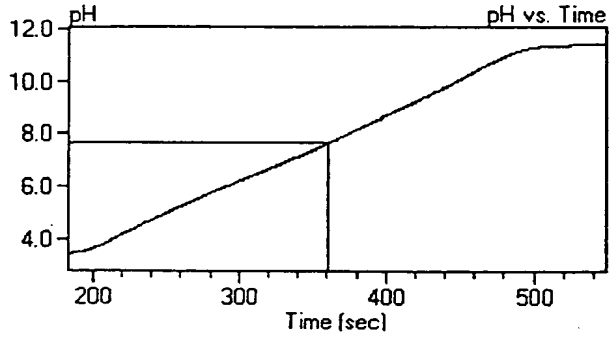



| X | Y | Fitted | Residual | Stdz Residual |
|---|---|---|---|---|
| 217.3 | 3.96 | 3.9554 | 0.0046 | 0.12 |
| 444.8 | 9.766 | 9.7419 | 0.0241 | 0.64 |
| 251.8 | 4.82 | 4.8329 | -0.0129 | -0.34 |
| 334.7 | 6.89 | 6.9415 | -0.0515 | -1.38 |
| 216.3 | 3.96 | 3.9299 | 0.0301 | 0.80 |
| 445 | 9.766 | 9.7470 | 0.0190 | 0.51 |
| 249.8 | 4.82 | 4.7820 | 0.0380 | 1.02 |
| 334.9 | 6.89 | 6.9466 | -0.0566 | -1.51 |
| 216.5 | 3.96 | 3.9350 | 0.0250 | 0.67 |
| 444.3 | 9.766 | 9.7292 | 0.0368 | 0.98 |
| 251.5 | 4.82 | 4.8253 | -0.0053 | -0.14 |
| 334.7 | 6.89 | 6.9415 | -0.0515 | -1.38 |

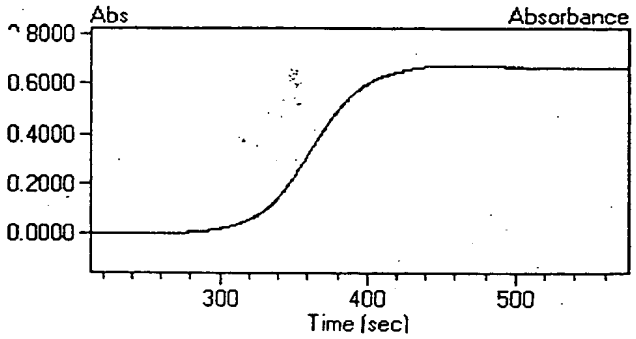
Results for data set: 18 - abs(4)

4-CN PHENOL



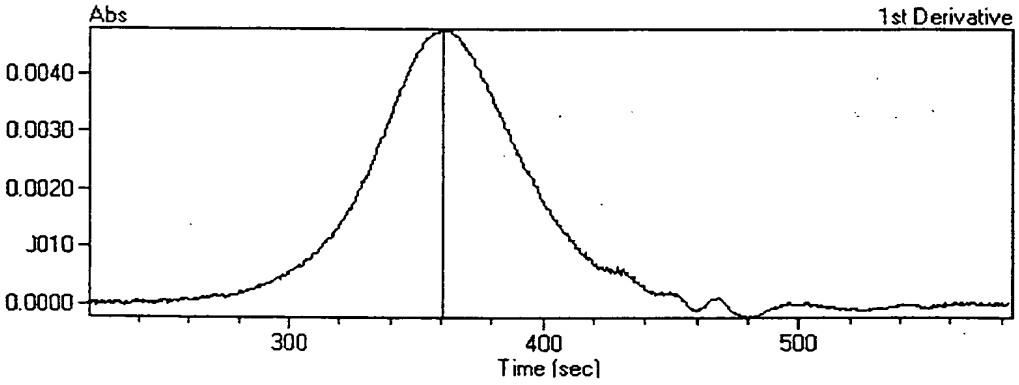

Calibration Curve

fig. 10




Absorbance Curve

fig. 11




First derivative of Absorbance

fig. 12

Time= 361.3 seconds

pH= 7.64

Figure 13 : Absorbance curve for an endpoint titration (KHP at 240nm)

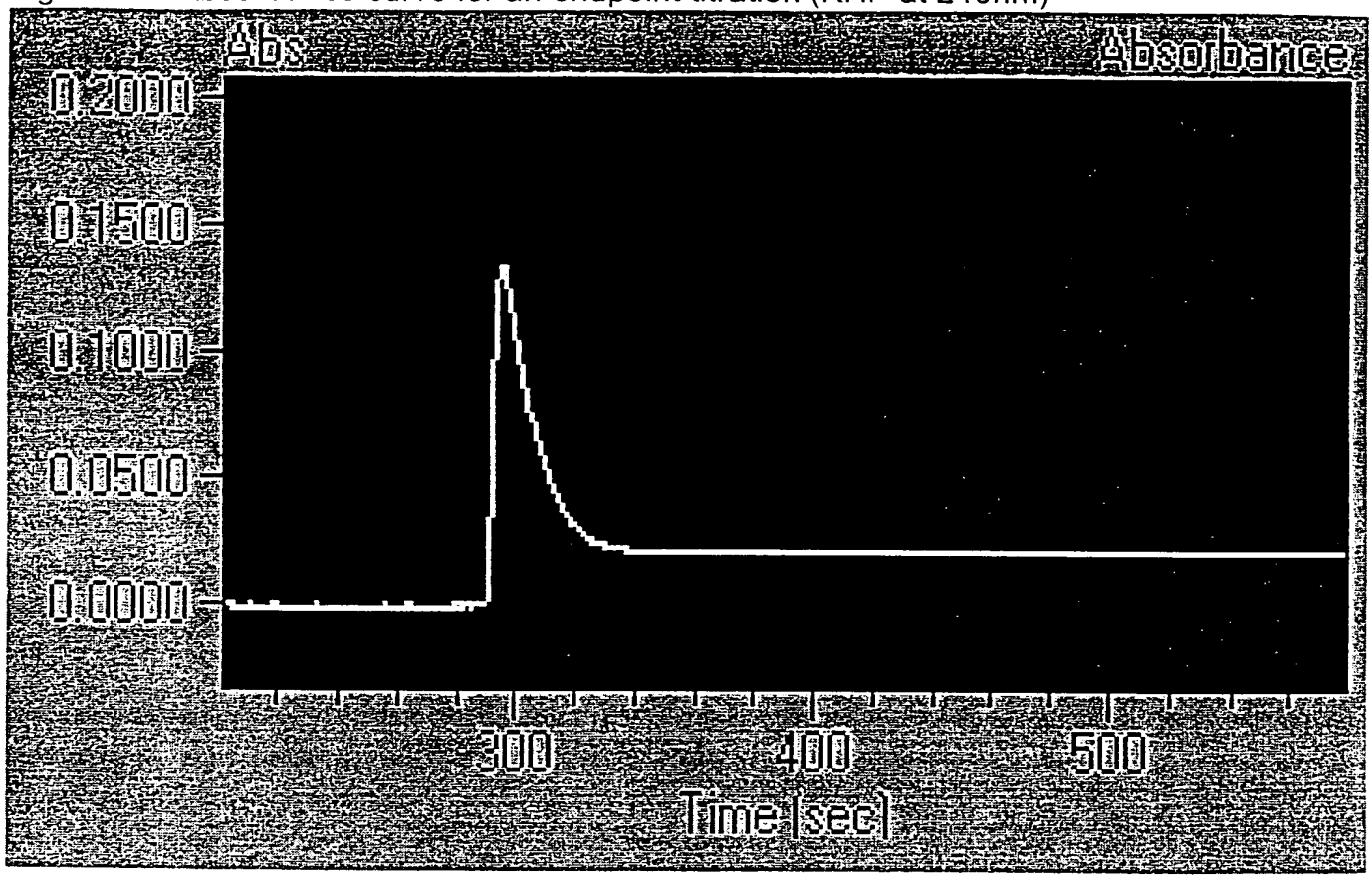


Figure 14: Calibration curve for endpoint titration(Potassium Hydrogen Phthalate)

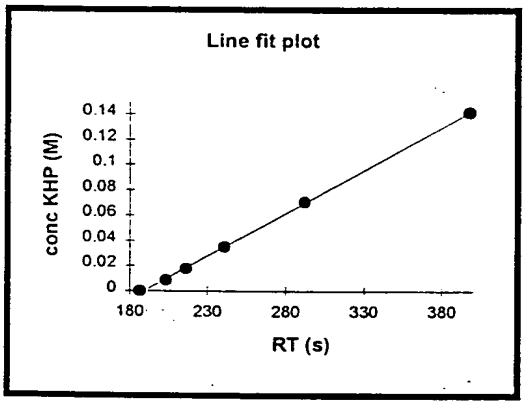
**Linear regression**

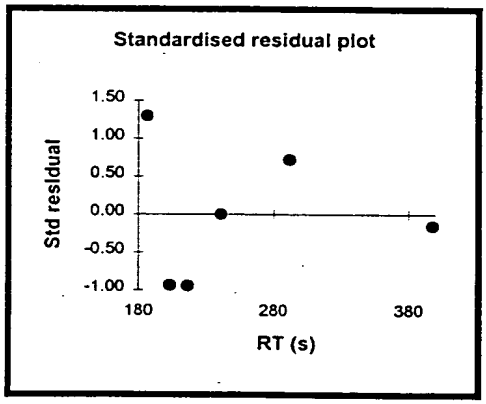
| | |
|---|---|
| | Determination of Potassium Hydrogen Phthalate concentration using continuous gradient titration with Phenolphthalein end-point indication |
| Performed By: | Alan Hill |
| Date: | 9-January-97 at 13:22 |
| Range: | [Accord1]Accord: RT (s) [5 - 10] v [Accord1]Accord: conc KHP (M) [5 - 10] |

| | |
|---|---|
| n: | 6 |
| R Square: | 0.9996 |

| | Coefficient | SE | *t* | *p* | 95% CI of Coefficient |
|---|---|---|---|---|---|
| Intercept: | -0.1278905 | 0.0018 | -72.1218 | <0.0001 | -0.1328 to -0.1230 |
| Slope: | 0.0006776 | 0.0000 | 101.6713 | <0.0001 | 0.0007 to 0.0007 |

| | Sum of Squares | *df* | Mean Square | *F* | *p* |
|---|---|---|---|---|---|
| Regression: | 0.01415 | 1 | 0.01415 | 10337.0546 | <0.0001 |
| Residual: | 0.00001 | 4 | 0.00000 | | |
| Total: | 0.01416 | 5 | | | |




| RT (s) | [KHP] M | Fitted | Residual | Stdz Residual |
|---|---|---|---|---|
| 186.5 | 0 | -0.0015 | 0.0015 | 1.30 |
| 398 | 0.1416 | 0.1418 | -0.0002 | -0.15 |
| 292 | 0.0708 | 0.0700 | 0.0008 | 0.72 |
| 241 | 0.0354 | 0.0354 | 0.0000 | 0.00 |
| 216.5 | 0.0177 | 0.0188 | -0.0011 | -0.94 |
| 203.5 | 0.0089 | 0.0100 | -0.0011 | -0.93 |

Fig 15.

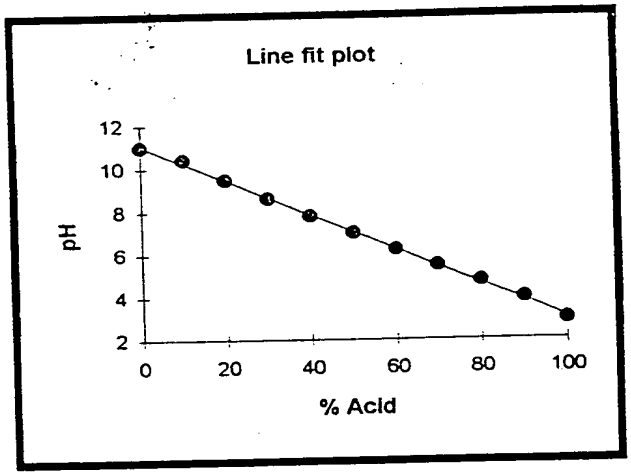
## Linear regression

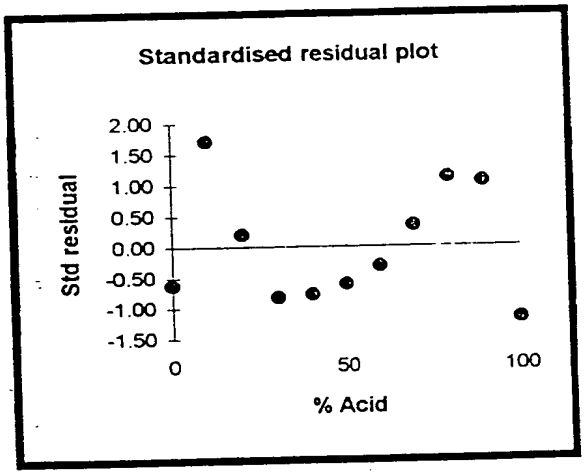
| | |
|---|---|
| Performed By: | New universal buffer 5cm3 min-1 flow rate<br>Alan Hill |
| Date: | 19-August-96 at 16:54 |
| Range: | [Sheet1]Sheet1: B [4 - 14] v [Sheet1]Sheet1: D [4 - 14] |

| | |
|---|---|
| n: | 11 |
| R Square: | 0.9991 |

| | Coefficient | SE | *t* | *p* | 95% CI of Coefficient |
|---|---|---|---|---|---|
| Intercept: | 11.0523 | 0.0468 | 235.9119 | <0.0001 | 10.9463 to 11.1583 |
| Slope: | -0.0804 | 0.0008 | -101.5630 | <0.0001 | -0.0822 to -0.0786 |

| | Sum of Squares | *df* | Mean Square | *F* | *p* |
|---|---|---|---|---|---|
| Regression: | 71.154 | 1 | 71.154 | 10315.0442 | <0.0001 |
| Residual: | 0.062 | 9 | 0.007 | | |
| Total: | 71.216 | 10 | | | |




| X | Y | Fitted | Residual | Stdz Residual |
|---|---|---|---|---|
| 100 | 2.91 | 3.0095 | -0.0995 | -1.20 |
| 90 | 3.9 | 3.8138 | 0.0862 | 1.04 |
| 80 | 4.71 | 4.6181 | 0.0919 | 1.11 |
| 70 | 5.45 | 5.4224 | 0.0276 | 0.33 |
| 60 | 6.2 | 6.2266 | -0.0266 | -0.32 |
| 50 | 6.98 | 7.0309 | -0.0509 | -0.61 |
| 40 | 7.77 | 7.8352 | -0.0652 | -0.78 |
| 30 | 8.57 | 8.6395 | -0.0695 | -0.84 |
| 20 | 9.46 | 9.4437 | 0.0163 | 0.20 |
| 10 | 10.39 | 10.2480 | 0.1420 | 1.71 |
| 0 | 11 | 11.0523 | -0.0523 | -0.63 |